UNIVERSITY OF PENNSYLVANIA

THE INJECTION OF CERTAIN SALTS INTO THE PROTOPLASM AND VACUOLES OF THE ROOT HAIRS OF LIMNOBIUM SPONGIA

THOMAS KERR

A THESIS

IN BOTANY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

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THE INJECTION OF CERTAIN SALTS INTO THE PROTOPLASM AND VACUOLES OF THE ROOT HAIRS OF LIMNOBIUM SPONGIA

By THOMAS KERR
(Botanical Laboratory, University of Pennsylvania)

With 5 Text-figures

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The role of the cations essential for growth in plant nutrition and their effects on protoplasmic structure and function are problems of first importance in plant physiology. Most of the results bearing on this have been obtained by immersing the plant parts in solutions of single salts or mixtures of various salts. In such immersion methods it is difficult to know whether the changes observed within the cell are caused by the entrance of the substances, by their effects on the surface, or by their action in causing the diffusion of substances from the cell.

The effects of salts on certain animal cells have been studied by injecting directly into the protoplasm. CHAMBERS and REZNIKOFF (5) injected the chlorides of Na, K, Ca and Mg into *Amoeba dubia* and found that the chlorides of Mg and Ca tend to precipitate or coagulate the protoplasm, whereas the chlorides of Na and K have a liquefying action. The same effect was also observed on starfish eggs (1) but EPHRUSSI and RAPKINE (8) did not get a similar action with Spirostomum and maintained that different results would be obtained with different cells. CHAMBERS and HOWLAND (3) working with *Actinosphaerium* and *Spirostomum* pointed out that the discordance in results may be explained by the difference in the way that the salts reach the protoplasm, according to whether the protoplasm is vacuolated or not. HEILBRUNN (14), by contrasting the ease with which cytoplasmic granules are sedimented by centrifuging cells immersed in various salt solutions, claims that Na and K raise the viscosity of protoplasm while Ca tends to lower it. This indicates that immersion and injection studies are not directly comparable.

The microinjection technique has not, as yet, been applied to plant cells. It seemed profitable, therefore, to use it to investigate the effects of the salts of K, Na, Mg and Ca on a typical plant cell and to determine whether the effects noted by CHAMBERS and REZNIKOFF can be duplicated upon plant material. The experiments described in this paper deal with the action of these salts upon the protoplasm and with the nature of the sap vacuole and the tonoplastic membrane.

MATERIAL AND METHODS

The plant cells here investigated were the young root hairs of the aquatic, *Limnobium Spongia* (BOSC.) RICH. This plant is closely related and similar to *Trianea bogotensis*, known also as *L. stoloniferum*, a form well known in physiological work because of the protoplasmic streaming of its large root hairs. These two plants have been confused by certain American horticulturists. *L. Spongia* is native to the southern United States, and is apparently quite variable. Its root hairs are 45—50 micra in diameter and the cells show rapid and regular protoplasmic streaming. A typical young specimen is shown in Fig. 1. The cytoplasm flows along the cell-wall from the base toward the tip, where it collects as it reverses its direction. In passing toward the base, the cytoplasm unites to form a central strand, which apparently traverses the sap-vacuole. The nucleus remains at the base of the root hair and can not be reached with a microneedle. The thin cell wall, protoplasmic streaming, low osmotic pressure, lack of plastids and other cell inclusions, make these root hairs ideal material for micrurgical investigation. The moist chamber and the technique of microinjection are those described by CHAMBERS (2).

The *Limnobium* roots were cut off from the plant and placed in a drop of pond water upon a large cover slip, which was inverted over a microdissecting moist chamber. In such a preparation the young root hairs showed normal streaming for at least 24 hours. The root hairs extend from the root in all directions, so that in a liquid drop preparation, a desired root hair was best reached by a micropipette of the vertical type, and the tip of the pipette generally punctured the lower surface of the cell. As a rule, only the younger root hairs were used for injection, inasmuch as these are rich in protoplasm and injury can, therefore, be more easily repaired.

When a microneedle or micropipette is brought up to the cell, it easily pierces the cell wall, much as a needle would pierce an inflated

balloon. The cell can be most easily pierced at the base, but a finely pointed pipette can puncture the hair at any place along the length provided the cell has not aged too much as to be more than 500 micra long. Longer root hairs are too tough and flexible and can only be punctured at their basal end. When a microneedle is inserted into a cell, streaming continues and the cell appears to be normal. The pipette can remain inserted indefinitely without causing any apparent harm, as long as precautions are taken to prevent jarring. When the pipette is removed, a plug of coagulated protoplasm is formed where the cell was pierced. This plug is similar to those noted by NICHOLS (21), SCARTH (26) and MARTENS and CHAMBERS (20) for various plant cells. Upon the removal of the pipette, streaming may stop momentarily but is soon resumed if the puncture is not too severe.

Operations were performed at all seasons of the year, and only healty root hairs were used. In these root hairs, protoplasmic streaming (i. e. cyclosis) is always seen under normal conditions, and the presence, type and rate of this cyclosis were taken as criteria for healthy cells. Cells which were injured as a result of injection either recover and regain their cyclosis at the end of an hour, or show positive evidence of death changes. These pathological conditions exhibited such symptoms as granulation and coagulation of the cytoplasm and nucleus, Brownian movement within the cytoplasm, and the formation of Ca oxalate crystals in the vacuole.

When a micropipette is inserted in a root hair, the pipette tip usually penetrates the vacuole (especially if the insertion is at the base). With a micropipette within the vacuole the internal pressure tends to press the vacuolar sap back into the pipette. Therefore, in order to inject liquids into the cell, a pressure must be exerted on the syringe of the injection apparatus, to overcome the considerable osmotic pressure of the cell. The streaming protoplasm is usually in greater amounts at the tip of the cell, and, if the wall is punctured in this region, the cytoplasm may itself be injected. Injections here must be made almost at the moment of piercing inasmuch as the cytoplasm tends to be pushed back into the pipette where it coagulates. The removal of the pipette from within the cytoplasm nearly always results in a cessation of the cytoplasmic movement away from the site of the plug. Thus, when the pipette is taken out of the cell tip, streaming in the direction of the base of the cell stops, but continues toward the tip, so that there is a heaping of the cytoplasm at the tip of the root hair. This massing of the cytoplasm lasts only a

few minutes. The amount injected from a micropipette can only be very approximately determined. Attempts were made to estimate the volume by comparing with the volume of a nucleus. On the average, twenty-five root hairs were injected with each concentration of salt studied.

The fact that injections can be both cytoplasmic and vacuolar, may best be illustrated in the case of the pH indicator, phenol red (4). This indicator, when injected into the vacuole slowly diffuses from the pipette, coloring the sap yellow. The cytoplasm remains colorless. If the indicator is introduced into the cytoplasm, the color is yellow with an orange tinge, while the sap remains colorless. Protoplasmic injections were made only into streaming protoplasm. There is an ectoplasmic layer of appreciable thickness which could not be injected directly.

LITERATURE

Root hairs have been extensively used in physiological work and much of the literature has been reviewed by Farr (9), (10). It is necessary here to review certain phases of the work which are concerned with this investigation. Aquatic root hairs, such as are present in *Limnobium*, *Trianea*, and *Hydrocharis* are unusually large in diameter, in contrast to the root hairs of land plants, and the nucleus of these aquatics is always in the base of the epidermis. In land plants the nucleus is frequently behind the apex of the hair.

Haberlandt (11) in 1887 first showed definitely that the root hair grows in length by the formation of additional material only at the apex, and this apical growth was later confirmed by Zacharias (30) and Reinhardt (27). Ziegenspeck (31) demonstrated that the cell wall at the tip is of a different chemical composition from that along the sides. The side walls give a cellulose reaction when treated chemically, whereas the tip stains blue with iodine and consists of a transitional carbohydrate, called amyloid. The apical growth of *Limnobium* root hairs can be easily demonstrated by making small punctures with a microneedle and following the position of the plug in the subsequent growth. This apical type of growth is in contrast to the type described by Ziegenspeck (32) in the closely related form, *Hydrocharis*. According to this author, *Hydrocharis* root hairs do not grow at the tip but deposit amyloid intercalarly near the base.

Ursprung and Blum (29) reported that the root hairs of *Vicia faba* show the lowest osmotic pressure of any of the cells of this plant. They

also state the osmotic pressure of root hairs depends to some extent on the medium in which they are grown. Root hairs already developed do not adjust themselves to the osmotic pressure of a new medium. Those, however, which grow after the root has been placed in the new concentration are adjusted to the new osmotic pressure. I have found that *Limnobium* root hairs in pond water possess a plasmolyzing value equivalent to 0.22—0.25 M. sucrose solution, a value fairly constant over long periods. The low osmotic pressure of these root hairs is an aid in microinjection.

Pfeffer (23) was one of the first to note the bursting of root hairs at their tip, when they were placed in hypotonic solutions. Zacharias (30) found that the cell wall might burst yet growth would continue with the formation of a new membrane. Klemm (18) found that low concentrations of acid have an explosive action on Trianea root hairs. He also made a study of the differences in the coagulative action of nitric and oxalic acids on *Trianea* protoplasm. Cholodnyj (6) worked on the effects of various mono- and bivalent cations on the protoplasm of *Trianea* and found that the alkali metals are toxic when the root hairs are immersed in single salt solutions. If the root hairs are placed in a solution of potassium salts, the movement of the protoplasm becomes slow and the protoplasm masses at the tip, giving one the impression of an increase in viscosity. This change gradually results in the death of the cell, but if the reaction has not gone too far, the cell may slowly recover on the addition of small amounts of calcium salts.

Hansteen-Cranner (12, 13), Coupin (7), Küster (19), Trelease (27) and Farr (10) all found that calcium is apparently necessary for root hair formation in all the forms studied. Hansteen-Cranner (12) found no correlation between the growth of the roots of wheat seedlings and root hair production; in fact the best growth of root hairs seemed to be in single salt calcium solutions. Kisser (17) demonstrated that root hairs would not grow in moist chambers if the seedlings were kept from coming in contact with calcium salts. In this case the roots grew, but no root hairs were formed. If the slightest trace of calcium was present, root hairs emerged. Both Hansteen-Cranner (12) and Farr (10) agree that the significance of calcium in the growth of root hairs seems to be that it is utilized directly in cell wall formation. Calcium is in fact the only constituent of the cell wall which is not produced within the plant.

EXPERIMENTAL

I. Immersion Experiments

It was found that *Limnobium* produces root hairs in solutions of calcium chloride in concentrations from 0.015 M. to 0.002 M. Mature plants were used in which root hair production was limited to the young adventitious roots. Growth, however, soon stopped and, after 6—8 days, the roots ceased to elongate and no further root hairs were formed. Root hairs were not produced when the plants were placed in distilled water or in solutions of either Na, K, or Mg chloride, and root hairs already present were dead at the end of 48 hours. Experiments in salt antagonism were not performed.

Root hairs of *Limnobium* which had grown in pond water behaved toward Na and K chloride, when placed in these solutions, essentially like that of *Trianea*, reported by Cholodnyj (6). If a root hair was placed in 0.02 M. NaCl, it showed a slackening in the rate of protoplasmic streaming and within two minutes contained a large mass of protoplasm, at the tip. Fully 80 % of the root hairs thus affected, burst at the tip without showing any increase in length. In the remaining root hairs there was a slow streaming for 10—30 minutes in which very little protoplasm took part in this streaming, as a large mass had accumulated at the tip. Gradually the protoplasm at the apex assumed the appearance of a dead coagulum.

II. Protoplasmic Injections

a) *Injections of distilled water.* A small or moderate amount may be injected into the streaming cytoplasm at the tip of the hair without stopping cyclosis. As the distilled water enters, it disperses the cytoplasmic granules around the site of injection. The mass of cytoplasm at this site bulges into the vacuole but quickly sinks down again as the dispersed granules flow down the cytoplasmic stream. If larger amounts are injected, the effect is sufficient temporarily to stop streaming in the direction of the base of the cell. This causes the protoplasm to mass at the tip. Although the distilled water disperses the granules, the accumulation of normal protoplasm in this region, and the possible exosmosis of water, cause the apical mass to appear normal within 90 seconds. At the end of three minutes, streaming is again renewed and the cell quickly recovers.

b) *Injections of NaCl and of KCl.* No differences could be noted between the effects of NaCl and of KCl. The effects of NaCl upon the protoplasm can best be illustrated by an account of a typical injection of 1.0 M. NaCl. A pipette filled with NaCl was inserted into the apex of the root hair, the tip of the pipette lying within the cytoplasm just at the point where the protoplasmic stream reverses its direction. When an amount of NaCl equivalent to the volume of the nucleus is introduced into the cytoplasm there are several immediate effects. The protoplasm and the salt mix freely and there is no evidence, whatsoever, of any coagulation of the cytoplasm. The vacuolar membrane bulges into the sap vacuole, the cytoplasmic volume increasing at the expense of the vacuole, for 10—20 seconds after injection has stopped. This increase is much more than can be accounted for by the amount injected and, therefore, one must assume that it is due to the entrance of water to establish an osmotic equilibrium. The protoplasmic streaming toward the base of the cell stops almost immediately, whereas movement toward the cell tip continues. This results in a massing of protoplasm at the apex of the root hair, within two minutes of injection. The protoplasm which comes from the base of the cell appears normal until it reaches the injected region, but here it mixes with the affected cytoplasm until a large mass, extending 150—200 micra from the tip, collects at the apical end of the cell (Fig. 2). At this time, there is a rapid Brownian movement of granules within this region, a condition never seen when massing is caused by a mechanical shock. The cytoplasm remains here without streaming for a period of 5 –10 minutes. Toward the end of this period the volume of the cytoplasm gradually decreases without any apparent loss due to streaming away. Coincident with this loss in volume, Brownian movement is no longer evident and the cytoplasm appears normal, while a churning movement now begins within the mass. Brownian movement continues longer in the protoplasm near the tip than in other parts of the cell. The decrease in volume is possibly due to the outward diffusion of the NaCl, either into the sap or to the outside and the attendant loss of water. About 10 – 15 minutes following injection, a thin stream of protoplasm gradually pushes out of the churning mass at the tip and flows to the base of the cell. Protoplasmic streaming is gradually renewed and the mass forms into strands, so that 30 minutes after injection the injected cells can not be differentiated from the controls.

In NaCl and KCl injections, it is necessary to keep the micropipette within the root hair until the massed protoplasm has moved from the

apex of the cell and the cell is recovering. If the pipette is removed immediately after the injection, most of the affected protoplasm flows out through the hole in the wall before plug formation occurs. If this is a small part of the total protoplasm, the hole becomes plugged, and the cell usually recovers. Should the pipette be removed just after the massing of the protoplasm at the tip, death invariably follows, since the protoplasm flows out without repairing the hole made by the pipette. Inasmuch as NaCl and KCl solutions adversely affect plug formation, the pipette must

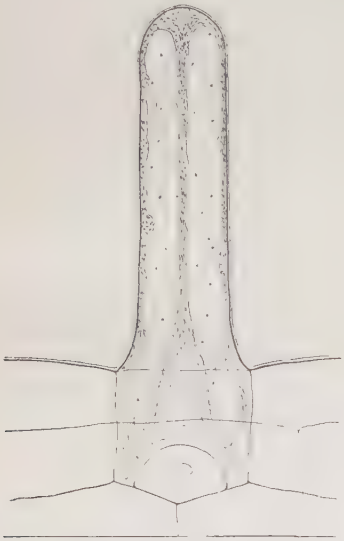

Fig. 1. Root hair of *Limnobium* showing normal strands of cytoplasm.

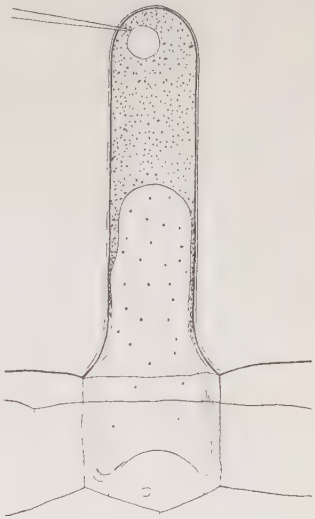

Fig. 2. Latent effect of m/1 NaCl two minutes after injection. A cytoplasmic vacuole frequently develops at the site of pipette tip some time after injection.

be kept in place until protoplasmic streaming has been renewed. It is plausible to assign the prevention of plug formation following Na and K injections primarily to their liquefying action.

This lack of plug formation following Na and K injections was also shown in the tendency of the injected solution and protoplasm to run back into the pipette. Such a back-flow is caused by the osmotic pressure within the cell. When a pipette with an aperture as small as 1 micron in diameter is used, the tip will clog. If the opening of the pipette is greater than 2 micra, there is usually a flow back into the pipette and a successful injection becomes difficult. However, protoplasm, which

has entered the pipette, may be injected back into the cell and if the pressure in the pipette is maintained for some time the cell will recover as previously described.

It has been possible, after an injection of NaCl or KCl, to take up the mixture of protoplasm and salt solution into the pipette, and inject this into the cytoplasm of a second root hair. The second root hair will show a typical sodium effect, and after recovery, the injected protoplasm becomes incorporated with the cytoplasm of the second cell. The added volume of cytoplasm is quite apparent in the increased material in cyclosis. This experiment has been performed successfully more than a dozen times and it has been found that a maximum amount of cytoplasm can be exchanged after a preliminary injection of 0.2 M. NaCl. If an amount of cytoplasm and salt mixture is injected directly into the vacuole instead of into the cytoplasm of a second cell the injected material becomes coagulated, gradually turns a dark brown color and persists as such in the vacuole.

When large amounts of concentrated NaCl or KCl solutions are injected, the cell dies, but death occurs only after rupture of the plasma membrane, either by the cell wall bursting at the tip of the cell, or around the region of the insertion of the pipette. The protoplasm then flows out as long as there is an internal pressure. The protoplasm that remains inside finally coagulates. In more than 200 injections of both NaCl and KCl, death never occurred unless the cell ruptured.

A break has never been observed in the vacuolar membrane, except when excessive pressure was exerted, no matter what concentration of NaCl or KCl was injected into the cytoplasm. This was checked by injecting a cell, the sap vacuole of which was colored by a previous injection of a dye, either phenol red or acid fuchsin, which remains in the vacuole without coloring the cytoplasm. When the salts were injected, the contour of the vacuolar membrane could easily be seen against the colored sap. The vacuolar membrane never broke and the protoplasm never became colored unless the entire cell burst. Thus we have a marked difference in the action of the outer and inner protoplasmic membranes; the outer membrane frequently breaking as a result of the NaCl or KCl injections, while the vacuolar membrane remains intact throughout.

In the injections of NaCl and of KCl, the intensity of the reaction is conditioned by the concentration used and the amount injected. Concentrations ranging from 2.0 M. to 0.05 M. of both Na and KCl were used. Even in the highest concentrations there was never an internal

coagulation of the cytoplasm, but injections of a nuclear volume of 2.0 M. resulted in the death of the cell in approximately half the cases by the breaking of the external membrane. Typical effects occur with diminishing intensity down to a concentration of 0.1 M., while with 0.05 M. the effects were so slight as to be indistinguishable from those of a distilled water injection. Thus it can be seen that these salts only produce a noticeable effect when injected into the cytoplasm in concentrations which would cause plasmolysis, were the root hairs immersed in such solutions.

It is a question whether the fluidity of the protoplasm seen upon NaCl and KCl injections is an osmotic effect or whether it is due to a specific action of these monovalent ions. The fact that this effect is only seen with concentrations greater than the osmotic pressure of the sap suggests that it is an osmotic phenomenon. Injections of concentrated dextrose and sucrose into the protoplasm produced a fluidity similar to that produced by K and Na chlorides. The protoplasm also increased in volume in a similar manner at the expense of the sap vacuole. Two essential differences were noted. First, during recovery after suger injections, the protoplasm usually became vacuolated. These vacuoles formed within the cytoplasm, a fact that could easily be seen by contrast when the sap was colored. Secondly, the cell did not fully recover for over an hour following dextrose injections, whereas normal streaming was present in less than 30 minutes when NaCl was used. The effects of dextrose are thus in agreement with the assumption that the fluidity produced by Na and KCl injections is at least in part due to osmotic action.

c) *Injections of* $CaCl_2$. The effects observed upon the injection of $CaCl_2$ into the protoplasm are almost the opposite to those produced with NaCl and KCl. Concentrations greater than 0.04 M. $CaCl_2$ will coagulate the cytoplasm. The protoplasmic plug at the region of insertion of the pipette forms with great ease after calcium injections, and the pipette may be removed immediately after injection with practically no loss of protoplasm. If the pipette is inserted at the apex of the cell, a deep brown coagulated mass forms immediately upon injection. Movement toward the base of the cell is usually stopped, and the cytoplasm accumulates as a thick viscous mass behind the coagulum (Fig. 3). If the coagulum is less than the size of the nucleus, streaming is renewed within 15 minutes and the cell recovers. The coagulated mass remains unchanged at the point where it is formed as long as the root hair remains alive. Frequently, when recovery begins, a mass of apparently normal cytoplasm surrounds the coagulum for a period of 10—30 minutes. This is usually

carried away by the stream as viscid lumps, which have very little movement of their own, but later disappear in the main mass of the streaming protoplasm.

In a normally streaming cell, only a localized region of cytoplasm around the pipette can be coagulated by the injection of calcium. Any further injection forces the calcium chloride through the tonoplast into the vacuole and not along the cytoplasmic stream. If the injection is made at the apex of the cell where the cytoplasm tends to collect, a maximum amount of cytoplasm can be coagulated. Here, if the coagulum is greater than the volume of the nucleus, death of the cell invariably follows. Such a death is a slow reaction. If, for example, the calcium has coagulated half the apex of the cell, movement will stop throughout, but within 30 minutes a definite movement will be resumed at the base of the hair. The part of the cytoplasm which still appears normal at the tip gradually coagulates until the whole apical end of the cell is discolored, while slight random movements may still be observed at the other end of the cell and may continue for as long as 3—4 hours.

When a concentration of 0.04 M. is injected a distinct coagulum results only with amounts greater than a nuclear volume. With concentrations from 0.04 M. to 0.005 M. the cytoplasm around the region of injection forms a jelly-like mass. The vacuolar membrane breaks easily in this region in contrast to the pronounced plasticity noted in the sodium injections. The break is followed by repair so that very little, if any, cytoplasm escapes into the vacuole. The chief evidence of the break is the formation of Ca oxalate crystals in the sap. During recovery from the injection of dilute concentrations, the reaction is the same but less severe than in the higher concentrations. Concentrations of $CaCl_2$ less than 0.005 M. only produce a distilled water reaction.

d) *Injections of* $MgCl_2$. $MgCl_2$, in concentrations of 1.0 M. and above, coagulates the cytoplasm if more than nuclear volume is injected. The coagulation is not accompanied by the immediate discoloration of the injected region nor is it as localized as the calcium effect. Concentrations less than M/1 were not found to produce coagulation, and below 0.04 M. there is no specific reaction to the salt. Solutions of M/1 $MgCl_2$ cause the protoplasm to set firmly around the region of injection. If the solution is introduced into the cytoplasm at the tip of the cell a preliminary dilution effect spreads down the stream for about 100 micra and the vacuolar membrane bulges into the sap vacuole as protoplasmic streaming toward the base slows down and ceases. The cytoplasmic granules very

gradually move into the injected area, and blister like excrescences appear, protruding into the sap vacuole. The dispersal of the granules throughout the injected region is not completed until 60—90 seconds after injection, and during this time, there is no plug formation should the pipette be withdrawn. The affected protoplasm at the site of injection does not exhibit Brownian movement, but the turgor of the vacuole may force the material down the pipette in the same manner as described for sodium injections. The normal streaming cytoplasm coming from the base does

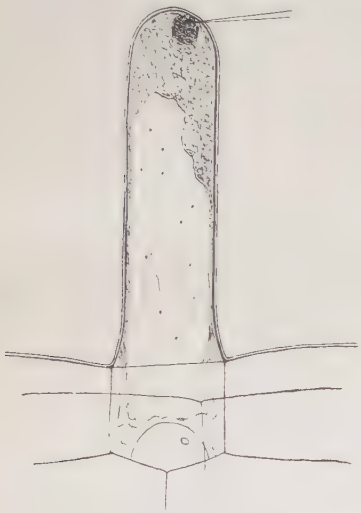

Fig. 3. The appearance of a root hair two minutes after injection of m/1 $CaCl_2$ showing coagulated area around the pipette tip.

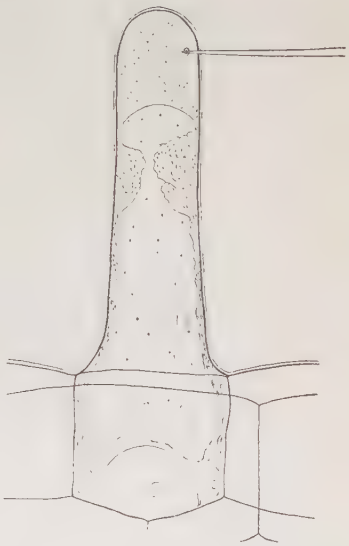

Fig. 4. Effect of m/1 $MgCl_2$ two minutes after injection, showing the cytoplasm from the base heaped up in masses distinct from the injected area.

not reach the affected region but heaps up in irregular masses behind the injected area (Fig. 4). After 15—20 minutes all the cytoplasm at the tip mixes, and, following this, there is another period of 10—15 minutes before normal streaming is renewed.

III. Vacuolar Injections

Injections into the sap vacuole can be made easily and in much larger amounts than into the cytoplasm. The injection of a colored solution shows that diffusion in the vacuole is practically independent of proto-

plasmic streaming. With a root hair 400 micra long, it takes fully 2 minutes for an injected substance to be evenly distributed throughout the cell.

The effects produced by the injections of NaCl, KCl and $MgCl_2$ are essentially alike. Considerable quantities of the salts in concentrations below 0.5 M. may be injected with no visible effect. The vacuolar membrane appears normal and streaming continues throughout and after the injection. Following the injection of M/1 NaCl nothing is to be noted until the amount within the sap vacuole is great enough to produce an osmotic effect. For example, when a volume equivalent to two nuclei is injected into the vacuole with a finely pointed pipette and the pipette withdrawn immediately, the protoplasm is not affected, and a plug forms at once.

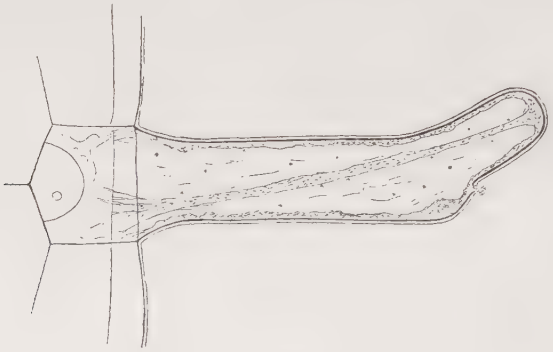

Fig. 5. Root hair showing apical growth 10 hours after it had burst at the tip following a vacuolar injection of m/1 NaCl.

The root hair begins to increase in length as the vacuole enlarges with a decrease in the rate of protoplasmic streaming. Frequently so much water is taken in that the root hair bursts at the tip and a certain amount of protoplasm is extruded. In approximately half the cases (over 50), a protoplasmic plug was formed and the cell recovered its streaming in spite of the loss of considerable protoplasm.

Occasionally growth occurred after the cell had recovered from bursting at the tip. This is shown in Fig. 5 which had grown 53 micra in length and still showed normal streaming ten hours after bursting at the tip. It is to be noted that the new growth took place to one side of the original tip of the cell. This demonstrates the apical growth of these root hairs.

If the cell does not burst, the increase in length due to swelling is usually permanent and streaming is normal within 5—10 minutes.

A significant phenomenon relating to the viscosity of the protoplasm is the shrinkage of the axial protoplasmic strand in the vicinity of the injected hypertonic solution, probably due to the dehydrating action of the latter. After the salt solution has diffused throughout the vacuole the strand recovers its normal appearance.

Calcium chloride injected into the sap vacuole, produces a shower of tetragonal crystals, indicating the presence within a normal vacuole of soluble oxalates which become precipitated as calcium oxalate. These crystals can be produced even with the injection of 0.002 M. $CaCl_2$. Although frequently seen in injured cells, these crystals do not occur normally in the vacuole of these root hairs. When the crystals are formed upon injection they drop to the lower side of the cell where they eventually sink through the vacuolar membrane into the cytoplasm in which they are carried around by the protoplasmic stream.

In order to observe the possible effect of immersion, *Limnobium* plants were placed in a 0.04 M. $CaCl_2$ solution for 24 hours, after which only the younger root hairs of the roots were alive and showed cyclosis. Calcium oxalate crystals were present in the dead cells but none whatsoever in the streaming root hairs. On the other hand injections of the 0.04 M. $CaCl_2$ into the vacuole, viz., the same solution in which the root hairs had been immersed for 24 hours, resulted in the usual shower of crystals.

With calcium injections, there is always a danger of damaging the strands of cytoplasm in the vicinity of the injection. It was found that once the oxalate had been precipitated in the vacuole, a further injection of $CaCl_2$ may coagulate the cytoplasm in the vicinity in much the same manner as if it were injected directly within the protoplasm.

If a root hair is cautiously given successive vacuolar injections of M/10 $CaCl_2$, it is possible to precipitate all the soluble oxalate within the vacuole without immediately coagulating the cytoplasm. Usually death results but in every case protoplasmic streaming always ceases to be renewed again in some cases at the end of an hour. Usually, however, it takes two hours for the cell to regain normal movement. At the end of this time, if calcium is again introduced into the vacuole, more oxalate crystals are precipated, indicating that, in the meantime, additional soluble oxalates had been formed. This suggests that either the presence of soluble oxalates or the absence of calcium within the vacuole is necessary for cyclosis within these root hairs. Saturated Na oxalate, injected into the vacuole, never produces a precipitate and does not stop protoplasmic streaming.

$CaCl_2$ was also injected into the root hairs of *Dianthus barbatus* and *Avena sativa*, both of which normally show a certain amount of protoplasmic streaming. This injection also was followed by the precipitation of calcium salt crystals. Osterhout (22) reported that crystals of Ca oxalate are formed when the root-hairs of *Dianthus* are placed in calcium solutions. He concluded from this that these root hairs absorb calcium ions. The writer has grown *Dianthus barbatus* seedlings in a chamber similar to that described by Farr (9) in which the root hairs were constantly immersed in an ever-changing solution of 0.01 M. $CaCl_2$. When undisturbed, these root-hairs grow in a manner similar to those of collards reported by Farr, and no crystals appeared within the vacuoles. However, a slight disturbance, such as transferring the seedling to a slide, resulted in the precipitation of Ca oxalate crystals within the vacuoles. This, apparently, is not toxic since the root hairs may continue growing.

LOCAL EXTERIOR APPLICATIONS

The apical bursting of cells showing apical growth has been described frequently in literature for root hairs, pollen tubes, and fungal hyphae immersed in hypotonic solutions exposed to substances which damage the delicate growing apex.

After a concentrated solution of NaCl has been injected into the vacuole, the root hair progressively elongates as the vacuole increases in volume until the root hair bursts at the tip. When the cells were immersed in 0.1 M. NaCl, most of the root hairs burst at the tip within 2 minutes without any increase in length.

If sodium oxalate is blown on the outer surface of a root hair within 15—20 micra of the apex the tip bursts quickly and violently. On the other hand, if the oxalate is applied locally against the side wall no bursting occurs but crystals appear within the pectin layer which forms the outer part of the cell wall. Frequently the pectic layer rises in the form of blisters. The crystals presumably are of calcium oxalate produced by interaction of the sodium oxalate with calcium pectate which Roberts (25) and Howe (16) found to be the composition of the outer layer of root hairs. At the same time the pectic layer becomes converted into soluble sodium pectate but the presence of cellulose maintains the required rigidity. At the apex of the cell the bursting is probably due to a similar demaging of the pectic layer. Evidently the amyloid apex described by Ziegenspeck (29) is not sufficient to hold up against the internal pressure

of the cell without the help of the pectic layer. It seems logical to assume that the bursting of the root hairs immersed in solutions of NaCl, is due to a similar action on the apex of the cell.

The bursting reaction of concentrated sodium salts into the vacuole is probably due in part at least to a different cause. The injection brings the salt into the vacuole where presumably it acts by increasing the internal osmotic pressure beyond the tensile strength of the apex of the cell.

DISCUSSION

It is not difficult to compare the effects of injecting various salts into *Limnobium* root hairs recorded here with the effects of injecting similar salts in amoebae and other animal cells described by Chambers and his co-workers.

Chambers and Reznikoff (5) observed that the monovalent NaCl and KCl salts produce a liquefying and quiescent effect upon amoebae. The same action was noted both when the amoebae were immersed in NaCl and KCl and also when the amoebae were injected with these salts. On the other hand Heilbrunn (14) claimed that sodium and potassium ions cause an increase in viscosity of several types of protoplasm and initiate coagulative changes while the ions of magnesium and calcium have the opposite effect. Heilbrunn quotes Cholodnyj's work (6) on *Trianea* as support for his conclusions. The effects which Cholodnyj observed in *Trianea*, I have also noted when *Limnobium* root hairs are immersed in NaCl solutions, viz., a decrease in the rate of streaming and the accumulation of the protoplasm at the apex of the cell. The accumulation of the protoplasm at the apex can be accounted for by the ready penetration of the salt at this region here exerting its specific quiescent effect. The streaming from the base continues for a time so that the protoplasm becomes massed at the tip. In the majority of the cells, bursting soon occurrs at the tip, while in the others, the material at the tip finally forms a dead coagulum. The fact that it is the protoplasm at the tip which is first affected agrees with the work of Chambers and Kerr (4) in which it was found that the cell wall at the apex is more readily permeable than along the sides of the root hair. The protoplasm was never observed to coagulate from injections of either weak or strong NaCl or KCl and recovery occurred as long as the outer membrane of the cell remained intact. After bursting coagulation does follow but this

surely can not be interpreted as the immediate action of NaCl or KCl. Chambers and Reznikoff also describe coagulation of amoeba dying when immersed in NaCl.

Several complicating factors must be considered in interpreting immersion experiments. It is well known that root hairs are normal only when kept in solutions containing calcium ions. True (28) quotes unpublished work of Miss Eckerson on the effect of placing wheat seedlings in solutions of K salts. Potassium rapidly penetrates and reacts with calcium pectate to form potassium pectate which dissolves in the water. At this stage, sugars, amino acids and salts, chiefly magnesium, diffuse rapidly out of the roots. A similar reaction occurs when the root hairs are placed in solutions of sodium salts in which case soluble sodium pectate is formed.

In an attempt to study viscosity changes by immersion methods one must take into account the complicating factors of the effect of salts on the cell wall and the exosmosis of various chemical substances. On the other hand an injection of NaCl and of KCl always produces the reverse of an increase in viscosity. However, it is still possible to explain the increased liquefaction to the osmotic action of the added salts which might more than counter-balance any possible coagulation effects. At any rate, there is no visible irreversible coagulation. NaCl and KCl are relatively non-toxic when injected into cytoplasm and exhibit definite liquefying effects only when in high concentrations. The fact that dextrose and sucrose show a similar liquefying effect when injected, supports this view.

The local coagulum produced by injections of $CaCl_2$ into the cytoplasm, is similar to that noted by Chambers and Reznikoff in amoebae. The amoebae are able to rid themselves of the coagulated mass by a pinching-off process, whereas the root hairs retain the coagulum within the rigid cell wall. The reaction of $MgCl_2$ is also similar since in both amoebae and root hairs, magnesium ions produce a general quiescence of the protoplasm and the affected cytoplasm apparently sets in the form of a gel. The concentration of the solutions injected to produce the typical salt effects are greater in the case of *Limnobium* root hairs than in amoebae, and are about of the same order as that in *Actinosphaerium*, (3). However, this may be largely due to the relative size of the organisms, the amoebae being much smaller than either the root-hairs or the *Actinosphaerium*.

The formation of a protoplasmic plug to close a puncture in the cell wall takes place with ease after calcium injections but with the greatest

difficulty after injections of NaCl, KCl and $MgCl_2$, and then only after the loss of considerable protoplasm. The plug consists of coagulated protoplasm and the difficulty of its formation after NaCl, KCl and $MgCl_2$ injections may be due not only to the lack of coagulation but also to an increase in volume of the cell following the imbibition of water. At the same time protoplasmic injections of NaCl and KCl frequently resulted in the rupture of the outer membrane while this rarely happened with $MgCl_2$ injections.

SUMMARY

By means of microinjection, the effects of the chlorides of K, Na, Ca and Mg were investigated upon the protoplasm and the vacuole of the root hairs of the aquatic plant, *Limnobium spongia*. This plant is closely related to the well-known *Trianea bogotensis* (*Limnobium stoloniferum*).

1. Root hairs develop in .015—.002 M. $CaCl_2$ but not in Na, K, or Mg chlorides which are toxic. In .02 M. NaCl the massing of the cytoplasm at the tip of the root hair is due to retardation of the streaming movement. After some time the majority of the root hairs burst at their tip. This does not occur when $CaCl_2$ is present.

2. Sodium chloride and potassium chloride are relatively nontoxic when injected into the protoplasm. These salts increase the fluidity of the protoplasm, but only when used in high concentrations (2.0 M. to 0.1 M.). This effect is interpreted as essentially an osmotic phenomenon. Dextrose and sucrose produce a similar state of fluidity of the protoplasm when injected in high concentrations. In lower concentrations (below 0.1 M.) injections of these salts produce no visible effect except to slow temporarily the protoplasmic movement. Death results from injections of NaCl and KCl only when the cell wall breaks.

3. Injections of $CaCl_2$ into the protoplasm in concentrations greater than 0.04 M. result in a local coagulation which persists, although the protoplast may recover its normal streaming with the coagulum remaining adherent to the cell-wall at the site of injection. Injections of $CaCl_2$ less than 0.04 M. and greater than 0.005 M. produce a local solidification of the protoplasm which is later incorporated.

4. $MgCl_2$, in concentrations greater than 0.1 M., when injected into the protoplasm, produces a widespread reversible solidification of the cytoplasm.

5. Injections of NaCl, KCl and $MgCl_2$ into the vacuole show no effect upon the cells except in sufficiently high concentrations to produce osmotic changes. Bursting of the cell apex may take place but this is frequently followed by recovery.

6. $CaCl_2$, when injected into the vacuole produces a shower of calcium oxalate crystals, indicating the presence of soluble oxalates within the sap. Cyclosis seems to be correlated with the lack of calcium ions in the sap of the vacuole in these root hairs.

7. Injections of high concentrations of sodium or potassium chloride into the cytoplasm causes the root hair to burst at the tip. The vacuolar membrane simply bulges into the vacuole. With calcium chloride the cell does not burst but the internal membrane breaks. This may be due mainly to their relative positions in the cell and to the respective weakening or stiffening action of the salts on the investing cell wall.

8. NaCl, KCl and $MgCl_2$ injections retard the formation of a protoplasmic plug at the site of puncture, while $CaCl_2$ apparently aids in its formation.

9. The increased fluidity of the protoplasm after an injection of NaCl or KCl makes it possible to withdraw some of the mixture of protoplasm and salt solution and to inject it into another root hair. When injected into the cytoplasm of a second cell, the injected material mixes with the protoplasm and becomes indistinguishable in the streaming. When injected into the vacuole, it persists as a coagulum.

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